Optimizing Contrast of Tip-Enhanced Fluorescence Microscopy for Imaging High-Density Samples

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Abstract: Quantum dots are imaged using tip-enhanced fluorescence microscopy. Optimization of the operation parameters leads to high-contrast images of high-density samples and a novel photon analysis improves contrast further.

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Various efforts in optical microscopy have been devoted to overcome the resolution limit imposed by classical light diffraction. Tip-enhanced fluorescence microscopy (TEFM) circumvents this limit down to ~10 nm resolution [1-3] by placing a sharp tip in the focus of a laser beam, leading to an enhanced optical field near the tip apex that can locally excite fluorescence. Most biological samples that could benefit from study with TEFM (e.g. membranes) are composed of a relatively high-density ensemble of various proteins and lipids, or other biomolecules. To study these samples, TEFM should be sensitive to individual molecules within the ensemble while maintaining nanoscale resolution. This is challenging because multiple molecules within the laser focus raise the background scattering level, thus lowering the signal-to-noise ratio (SNR) and reducing the sensitivity. Thus, to apply TEFM to real biological systems, it is crucial to optimize the contrast.

In our scheme, a green He-Ne laser beam (λ=543 nm) was introduced into an inverted microscope equipped with a high numerical-aperture objective and a polarization converter, producing an axial field within a tight focus spot. An atomic force microscope (AFM) tip was aligned into the focus spot to locally enhance the incident field. The emitted fluorescence signal, containing both far-field and near-field components, was collected with the same objective, spectrally filtered and focused onto an avalanche photodiode. The AFM probe was oscillated in tapping mode at a frequency of ~70 kHz. This modulated the fluorescence rate, and the near-field signal was demodulated with a lock-in amplifier [2, 4]. Fig. 1(a) shows a fluorescence photocount image of a high density sample of CdSe-ZnS core-shell quantum dots (QDs), while Fig. 1(b) shows the same data after demodulation by a commercial lock-in. Clearly, the SNR of the lock-in data is much larger than for the simple photon sum image. Using the lock-in, the SNR of single QDs within ensembles of increasing density were measured and individual particles were still easily resolved (SNR > 5) at a density of ~15 QDs/μm^2 [2]. Figure 2(c) shows the image of a single isolated QD and the signal profile along the indicated axis. The full width at half maximum is about 12 nm, which approximates the radius-of-curvature of the probe apex as determined from the topography image (data not shown). The width of the signal profile suggests a spatial resolution near 10 nm, limited only by the probe sharpness.

Time-correlated single photon counting (TCSPC) is a powerful technique for acquiring fluorescence data whereby the precise arrival time of each detected photon is recorded permanently to a computer disk [1, 5]. A time marker corresponding to a particular phase in the oscillation cycle of the AFM tip is also recorded and the photons can be correlated to the instantaneous phase of the tip oscillation. Ultimately the phase correlation can be mapped

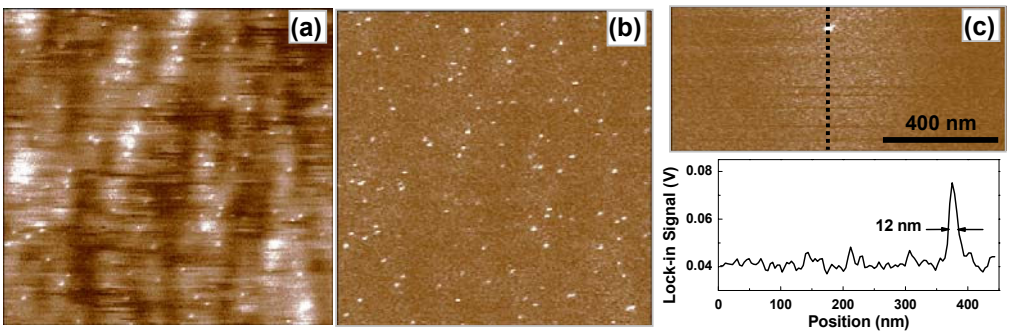

Fig.1. (a) Photon sum image of a high-density sample of QDs, scan size 5 × 5 μm^2; (b) Demodulated image by a lock-in amplifier; (c) High-resolution image of a single QD and signal profile along the specified axis.

onto the instantaneous tip-sample separation to produce near-field tomography images [4]. This data acquisition scheme imparts complete flexibility in applying various data analysis algorithms and we have begun to investigate its use for improving TEFM contrast yet further. Figure 2(a) is a 1×1 μm^2 fluorescence photocount image of a pair of QDs. TCSPC allows us to record a histogram of photon phase delays (relative to the tip oscillation) for any region of an image, as shown in Fig. 2(b). We identify the red-boxed area as the enhanced region and the peak of the histogram sets the enhanced phase (the optimal lock-in phase), which is around 60° in this case. The green region is 180° out of phase corresponding to the background. Figure 2(c) gives the results of a digital lock-in algorithm applied within our analysis software and Fig. 2(d) shows that the lock-in method is basically a vector addition by considering each photon as a unit vector in phase space.

Since our analysis technique gives access to the phase information of each photon, we can apply various additional phase filters before applying the lock-in algorithm. Figure 2(e) shows the results of a "binned lock-in" algorithm that offers moderately improves contrast over a standard lock-in. As shown in Fig. 2(f), photons that fall outside the phase windows determined by the red and green shaded regions are rejected prior to applying the standard lock-in algorithm. The width of these windows can be optimized for a particular data set as dictated primarily by the cantilever oscillation amplitude [2]. Figure 2(g) shows the results of a "weighted lock-in" algorithm: the vector "lengths" of photons within the data windows are scaled according to a weighting function that is determined by the shape of the histogram in Fig. 2(b). This algorithm is shown schematically by the phase-space diagram in Fig. 2(h). Monte-Carlo simulations of our experiment indicate that each of these additional analysis methods must be optimized by varying the AFM-tip oscillation amplitude and the width of the phase windows. The simulations also suggest that such optimization can lead to an ~30% improvement in contrast relative to a standard lock-in algorithm for isolated fluorophores and that this advantage increases with increasing fluorophore density.

We have shown that tip-enhanced fluorescence microscopy can be used to image high-density fluorophore samples and that contrast can be improved significantly by optimization of the AFM operation parameters and through the use of a novel photon analysis method. These results have favorable implications for the eventual nanoscale imaging of viable biological samples, such as cellular membranes.

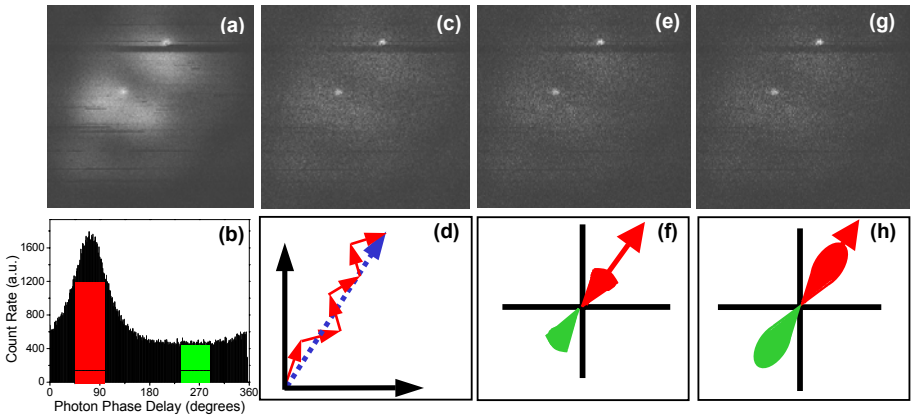

Fig.2. (a) Photon sum image; (b) histogram of photon phase delays; (c) lock-in image; (d) phase-space schematic of a lock-in algorithm; (e) binned lock-in image; (f) phase-space schematic of a binned lock-in algorithm; (g) weighted lock-in image; (h) phase-space schematic of a weighted lock-in algorithm.

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